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Selection of spring bread wheat genotypes for resistance to cereal cyst nematode (*Heterodera avenae* Woll.) based on field performance and molecular markers

Khaled A. Moustafa[1,2], Abdullah A. Al-Doss[1], Mohamed I. Motawei[3*], Solaiman Al-Otayk[3], Ahmed A.M. Dawabah[1], Ahmed L. Abdel-Mawgood[1], Suloiman M. Al-Rehiayani[3], and Ahmad S. Al- Hazmi[1]

[1]College of Food and Agricultural Science, King Saud University, Saudi Arabia
[2]Agricultural Research Center, Field Crops Research Institute, Egypt
[3]Plant Production and Protection Department, Al-Qassim University, Saudi Arabia

***Corresponding author: rumotawei@hotmail.com**

Abstract

The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is a devastating root nematode parasite of wheat (*Triticum aestivum* L.). This study aimed to screen wheat germplasm for resistance to CCN. The performance of 17 genetically diverse wheat genotypes (local and international material) were evaluated for two years (2009 and 2010) in a *H. avenae*-naturally-infested field at the Hial region, north Saudi Arabia. Results show that the tested wheat genotypes were significantly different in field performance and resistance to CCN. The grain yield ranged from 4.58 tons/ha for cv. Yecora Rojo (the susceptible) to 8.2 tons/ha for the genotype 15-SAWYT-31. Ten local genotypes were designated as resistant. The local cv. KSU 119 was the most resistant genotype (no. cysts/plant = 0.7) among all the genotypes tested. In addition, microsatellite markers linked to *Cre1* and *Cre3* genes were used. The dendogram generated using SSR data divided wheat genotypes into two main clusters. Ten out of 17 wheat genotypes (LNM-72, LNM-99, LNM-126, LNM-136, KSU118, L11-8, L11-17, L11-21, KSU 119, and AUS-30851) had both *Cre* genes and were found in the same sub-cluster. All these genotypes, except AUS-30851, LNM-72 and L11-17, were found to be the resistant to CCN. Therefore, *Cre3*, *Cre1* and other *Cre* resistance genes are now used in our marker-assisted selection (MAS) programs to identify CCN-resistant wheat genotypes.

Key words: Wheat, *Heterodera avenae*, Pathotypes, PCR, Marker Assisted Selection (MAS).
Abbreviation: CCN_ cereal cyst nematode, MAS_ marker-assisted selection, RFLP_ restriction fragment length polymorphism, SSR_simple sequence repeats, GY_ grain yield, BY_ biological yield, PH _plant height, HI_ harvest index, CTAB_cetyltrimethyl−ammonium bromide, TAE_Tris-acetate-EDTA, ANOVA_analyses of variance, UPGMA_Unweighted Pair-Group Method with Arithmetical Averages.

Introduction

Cereal cyst nematode (CCN), *Heterodera avenae* Woll., has a global distribution and causes significant economic yield losses to cereal crops in many countries of the world (Nicol et al., 2003; Ogbonnaya et al., 2001). Developing high yielding cultivars with tolerance to abiotic and biotic stresses is one of the main challenges to wheat breeders. In light of the new information on CCN, we are interested in incorporating nematode resistance genes into new high yielding cultivars of wheat. This would assist in 2nematode control, since it is cost-effective and friendly to the environment. Eight genes that confer resistance to CCN have been identified in hexaploid wheat and its relatives (Ogbonnaya et al., 2001). These are *Cre1* (Cereal root eelworm—locus 1) (*Triticum aestivum* 2B) (Williams et al., 1994), *Cre2* (transferred to wheat from *Aegilops ventricosa*) (Delibes et al., 1993), *Cre3* (2D, transferred from *Aegilops tauschii*) (Eastwood et al., 1994), *Cre4* (*A. tauschii*) (Eastwood et al., 1991), *Cre5* (2A, VPM1 segment from *A. ventricosa*) (Jahier et al., 2001), *Cre6* (*A. ventricosa* 5NV) (Ogbonnaya et al., 2001), *Cre7* (*Aegilops triuncialis*) (Romero et al., 1998) and *Cre8* (*T. aestivum* 6B) (Williams et al., 2003). CCN resistance genes have also been mapped in rye (*6R*, Taylor et al., 1998) and barley (*2H*, Kretschmer et al., 1997).

A linkage disequilibrium study has also found an RFLP locus, Xcdo347, which was associated with the Festiguay-derived CCN resistance of the wheat cultivars Molineux, Frame and Barunga (Paull et al.1998). Williams et al. (2003) used this RFLP as a starting point to genetically locate the gene *Cre8* which provides CCN resistance (and tolerance) in the cultivar Molineux. *Cre1* confers resistance to several European *H. avenae* pathotypes as well as the Australian pathotype, albeit with varying levels of nematode reproduction in different genetic backgrounds of the host (Majnik et al. 2003). Comparison of *Cre1* with the CCN resistance gene *Cre3*, derived from the diploid D genome progenitor of wheat, *Aegilops tauschii*, showed that both provide resistance to the Australian pathotype, but differed in their specificity to European and Middle Eastern pathotypes (Ogbonnaya et al., 2001). The purpose of this research is to: (i) identify wheat genotypes that high yielding under the stress of *H. avenae* infection in the field, (ii) screen wheat genotypes for resistance to CCN under field conditions, and (iii) correlate wheat genotypes with molecular markers for *Cre1* and *Cre3* genes to CCN resistance in the field and harvest yield.

Table 1. Names and pedigrees of 17 wheat genotypes tested in NADC wheat farms at Hail.

NO	Name	Pedigree	Origin
1	LNM-72	Wheat line selected from an off-type plant	Plant Production Department
2	LNM-99	Wheat line selected from an off-type plant	Plant Production Department
3	LNM-126	Wheat line selected from an off-type plant	Plant Production Department
4	LNM-136	Wheat line selected from an off-type plant	Plant Production Department
5	15 SAWYT- 30	CIMMYT -WORRAKATTA/2*PASTOR	CIMMYT breeding program
6	15 SAWYT- 31	CIMMYT -WORRAKATTA/2*PASTOR	CIMMYT breeding program
7	15 SAWYT- 38	CIMMYT -TIE CHUAN1*2/3/HE1/3*CNO79//2*SERI	CIMMYT breeding program
8	15 SAWYT- 42	CIMMYT -CROC_1/AE.SQUARROSA (205)//BORL95/3/KENNEDY	CIMMYT breeding program
9	KSU 118	Sama\ Yecora Rojo-L10-1	Plant Production Department
10	KSU 110	Sama\ Yecora Rojo-L11-6	Plant Production Department
11	L11-8	Sama\ Yecora Rojo-L11-8	Plant Production Department
12	L11-17	Sama\ Yecora Rojo-L11-17	Plant Production Department
13	L11-21	Sama\ Yecora Rojo-L11-21	Plant Production Department
14	KSU 114	Sama\ Yecora Rojo-L11-23	Plant Production Department
15	KSU 119	Sama\ Yecora Rojo-L10-4	Plant Production Department
16	AUS-30851	AUS 4930.7/2* PASTOR	Australian breeding program
17	Yecora Rojo	The recommended cultivar	USA

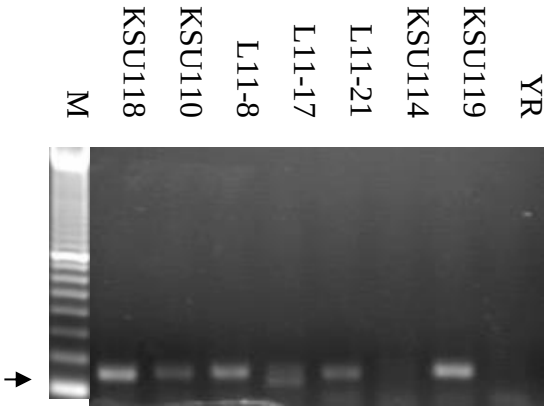

Fig 1. Polymorphism revealed using SSR primers (Xgwm301) to amplify genomic DNA purified from wheat genotypes. M lane is 1 kbp ladder DNA marker. Arrow shows the polymorphic band.

Results and Discussion

Field performance of selected wheat genotypes

Grain and biological yields of all wheat genotypes were higher ($P \leq 0.05$) than those of cv. Yecora Rojo, which is the susceptible standard and the principle cultivar grown in Saudi Arabia (Table 2). The grain yield ranged from 4.58 tons/ha for cv. Yecora Rojo to 8.2 tons/ha for the genotype 15-SAWYT- 31 (Table 2). The cv. Yecora Rojo has previously been shown to be sensitive to the Saudi populations of *H. avenae* (Al-Hazmi and Dawabah, 2009; Al-Hazmi et al., 1994; 1999; Ibrahim et al., 1999). Wheat yield depends on interacting ecological and edaphic factors, but it is highly responsive to *H. avenae* infection, even when other stresses restrict yield (Smiley et al., 2005). This finding has been supported by research in Saudi Arabia that showed a reduction in grain and straw yields of wheat is proportional to *H. avenae* densities in the soil, either in pot experiments (Al-Hazmi et al., 1999) or under field conditions (Ibrahim et al., 1999). *H. avenae* infection disturbs several physiological aspects of infected wheat plants, such as photosynthesis, mineral uptake, transpiration and water content of leaves and roots (Al-Yahya et al., 1998). In the present study, Saudi genotypes including KSU 110, L11-8, L11-17, L11-21, KSU 114 and KSU 119, were characterized by short height (Table 3). All of these cultivars, except L11-17, were resistant to *H. avenae* infection.

Screening wheat genotypes for resistance to CCN

Among the tested wheat genotypes, 10 local genotypes (selected from the wheat breeding program, Plant Production Department, King Saud University) were designated as resistant to CCN (Table 3). On the other hand, CIMMYT genotypes (15 SAWYT-30, 15 SAWYT-31, 15 SAWYT-38 and 15 SAWYT-42) plus the cvs. AUS-30851 and Yecora Rojo were found to be the most susceptible genotypes to the tested Saudi population of *H. avenae*. The significant negative correlations of number of cysts/root with biological yield and plant height (Table 4) indicates the importance of resistant genotypes in improving biological yields. On the other hand, the correlation between number of cysts/root and grain yield ($r = 0.06$) was not significant. Microsatellite markers linked to *Cre1* and *Cre3* genes were used in this study (Majnik et al. 2003; Martin et al. 2004). The dendrogram generated using SSR data divided wheat genotypes into two main clusters (Fig. 2). The first cluster was divided into two sub-clusters that included the following: (i) LNM-72, LNM-99, LNM-126, LNM-136, KSU 118, L11-8, L11-17, L11-21, KSU 119 and AUS-30851, and (ii) 15 SAWYT-38 and KSU 110. The second cluster consisted of

Table 2. Agronomical traits of 17 wheat genotypes in a *Heterodera avenae* naturally-infested field in NADEC experimental station at Hail in two seasons (2009 and 2010).

Genotypes	Grain yield (Ton/ha)	Biological yield (Ton/ha)	Harvest index (%)	Plant height (cm)
LNM-72	6.86	20.8	0.33	108.3
LNM-99	5.59	22.0	0.26	112.2
LNM-126	5.88	19.7	0.30	104.0
LNM-136	5.83	21.2	0.28	111.3
KSU 118	6.30	15.9	0.41	87.7
L11-8	5.96	15.8	0.39	75.7
L11-17	5.99	15.9	0.38	67.3
L11-21	6.78	17.1	0.39	75.5
KSU 119	5.59	14.7	0.39	72.5
AUS-30851	7.67	18.9	0.41	81.0
KSU 110	7.18	16.2	0.45	69.3
15 SAWYT- 38	7.47	20.1	0.39	105.0
KSU 114	5.95	15.1	0.40	68.2
15 SAWYT- 30	7.63	18.5	0.42	82.8
15 SAWYT- 42	6.68	15.9	0.41	88.2
15 SAWYT- 31	8.20	21.1	0.38	81.3
Yecora Rojo	4.58	10.2	0.45	58.2
LSD 5%	1.24	3.2	0.05	7.5

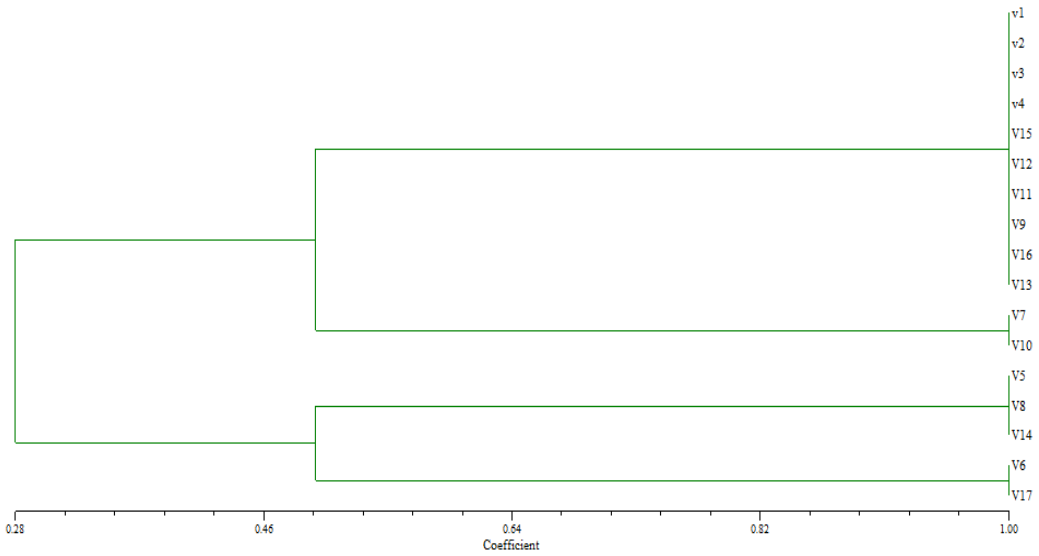

Fig 2. Dendrogram constructed from similarity coefficients and showing the clustering of wheat genotypes (V1= LNM-72, V2= LNM-99, V3= LNM-126, V4= LNM-136 V5= 15 SAWYT- 30, V6= 15 SAWYT-31, V7= 15 SAWYT-38, V8= 15 SAWYT-42, V9= KSU 118, V10= KSU 110, V11= L11-8, V12= L11-17, V13= L11-21, V14= KSU= 114, V15= KSU 119, V16= AUS-30851, and V17= Yecora Rojo).

two sub-clusters that included the following: (i) 15 SAWYT-30, 15 SAWYT-42 and KUS 114 and (ii) 15 SAWYT-31 and Yecora Rojo. Compared to the other genotypes, the last genotypes 15 SAWYT-31 and Yecora Rojo were among the most susceptible genotypes. Ten out of 17 wheat genotypes (LNM-72, LNM-99, LNM-126, LNM-136, KSU118, L11-8, L11-17, L11-21, KSU 119, and AUS-30851) had both *Cre* genes and were also found in the same sub-cluster (Fig. 2). These genotypes, except AUS-30851, LNM-72 and L11-17, were resistance to CCN. L11-17 had two bands for *Cre3* primers (Fig. 1) and was susceptible genotype (3.49 cysts/plant) (Table 3). Genotypes KSU 110, KSU 118, KSU 119, L11-8, 1nd L11-21 had only one band for *Cre3* primers (Fig. 1). Therefore, these genotypes were homozygous for *Cre3* gene and were resistance genotypes. AL-Ghumaiz and Motawei (2011) suggested that co-dominant SSR polymorphism can be detected without the time consuming usually associated with SSR development and characterization. The *Cre3* gene in the wheat genotypes as detected by Xgwm301 (Fig. 1) provided high levels of resistance to CCN. However, the Australian genotype (AUS-30851) had both *Cre* genes and was among the most susceptible genotypes. The possible explanations of this result may be that the Saudi genotypes had more *Cre* resistance genes toward *H. avenae* (Monetes et al., 2008). On the other hand, CIMMYT genotype (15 SAWYT-31) and the cv. Yecora Rojo did not have both *Cre* genes and were found to be the most susceptible genotypes. Majnik et al. (2003) reported that the tight linkage between csAtPPR5/BCD1231 with the *Cre1* and *Cre3* loci, suggesting a homeolocus relationship. In addition, overlapping resistance specificity between *Cre1* and *Cre3* against *H. avenae* pathotypes have been reported (Rivoal et al. 2001). Earlier reports showed that the gene-based SSR marker (Xgwm301) have the most significant association explaining 27% of the phenotypic variation in CCN resistance amongst the wheat genotypes

Table 3. Screening 17 wheat genotypes for *Cre* genes and number of cysts/root system.

Genotypes	***Cre1***	*Cre 3*	No. cysts/plant	Reaction
LNM-72	+	+	3.22	Susceptible
LNM-99	+	+	0.31	Resistant
LNM-126	+	+	1.34	Resistant
LNM-136	+	+	0.96	Resistant
KSU 118	+	+	2.31	Resistant
L11-8	+	+	2.29	Resistant
L11-17	+	+	3.49	Susceptible
L11-21	+	+	1.77	Resistant
KSU 119	+	+	0.67	Resistant
AUS-30851	+	+	9.19	Susceptible
KSU 110	-	+	2.17	Resistant
15 SAWYT- 38	-	+	18.48	Susceptible
KSU 114	+	-	1.41	Resistant
15 SAWYT- 30	+	-	27.52	Susceptible
15 SAWYT- 42	+	-	22.76	Susceptible
15 SAWYT- 31	-	-	21.34	Susceptible
Yecora Rojo	-	-	27.74	Susceptible
LSD 5%			10.66	

Table 4. Simple correlation coefficients among agronomical traits and number of cysts/root system of 17 wheat genotypes.

Trait	Grain yield	Biological yield	Harvest index	Plant height	number of cysts/root
Grain yield	1.00				
Biological yield	0.83**	1.00			
Harvest index	0.12	-0.44**	1.00		
Plant height	0.29*	0.60**	-0.59**	1.00	
Number of cysts/root	-0.06	-0.21*	0.29*	-0.20*	1.00

*, ** Significant at P=0.05 and 0.01, respectively.

(Al-Doss et al. 2009; Al-Doss et al. 2010). Also, there was significant association between CCN resistance and *Cre1* locus which explained 12% of the phenotypic variation (Al-Doss et al. 2009). Therefore, amplification conditions for the Xgwm301 marker were used in marker-assisted selection to identify *Cre3* CCN-resistant wheat in the Saudi wheat cultivars. The gene-based STS marker, *Cre3sp* on chromosome 2DL showed the most significant association explaining 23% of the phenotypic variation in CCN resistance (Ogbonnaya et al. 2001). Our results indicate that neither *Cre1* or *Cre3* genes were found in the genetic background of Yecora Rojo. However, in our results, nine advanced genotypes selected from the wheat breeding program at King Saud University, were amplified with the *Cre1* and *Cre3* primers. Combining the *Cre1* and *Cre3* primers in one assay would be highly cost- and time-effective; however, this requires more detailed studies. This study clearly shows that markers can be optimized and utilized for characterizing genotypes. Also, further work is needed to assess the other microsatellite markers linked to *Cre* resistance genes in the Saudi genotypes. These markers will be applied in a marker assisted selection of *Cre* genes in early generation which are otherwise complex and expensive to phenotype within a breeding program. As Ogbonnaya et al. (2001) indicated that the traditional screening method presents a high degree of variation, and efforts are underway to improve the assay accuracy, to further validate the current findings.

Materials and Methods

Screening field performance of wheat genotypes

Seventeen genetically diverse wheat genotypes were planted in two successive growing seasons (2008/2009 and 2009/2010) in a *H. avenea*-infested field in Nadec company, Hail, North Saudi Arabia. These included 11 genotypes selected from the wheat breeding program at the Plant Production Department, College of Food and Agriculture Sciences, King Saud University, four genotypes selected from CIMMYT breeding program, one selected genotype from the Australian breeding program and the recommended cultivar Yecora Rojo (Table 1). In each season, a nematode-infested site was selected in the field and divided into small plots in a randomized complete block design with three replicates. The plot dimensions were 3 m long and 0.8 m wide with row-to-row spacing of 20 cm. Planting was carried out at the last week of December, with a seeding rate of 140 kg/ha. The recommended fertilizer requirement for wheat in Hail region (200, 200 and 100 kg/ha NPK, respectively) was applied. Plant samples were carefully collected one month before harvest, keeping intact as much of the root systems as possible. Roots were freed of soil using a gentle stream of tap water over a 60 mesh sieve. The clean roots were examined for the presence of white cysts. The total numbers of white cysts per soil and root system were determined and the average number of white cysts per plant was calculated. Plants having more than three cysts per plant were designated as susceptible, while those having up to three cysts per plant were designated as resistant (Mathur et al., 1974; Irohlm, 1994). At maturity, grain yield (GY), biological yield (BY) and plant height (PH) were determined. Grain and biological yields were also determined for each plot and converted to weight per hectare. Harvest index (HI) was calculated as grain yield/biological yield.

Molecular analyses

Young leaves (500 mg of each genotype) were frozen and ground to a powder in a mortar with liquid nitrogen. The powder was poured into tubes containing 9.0 ml of warm (65°C) cetyltrimethyl−ammonium bromide (CTAB)

extraction buffer (1.5M NaCl,100 mM Tris-HCl pH 8, 20 mM EDTA, 1% CTAB) (Sagahi-Maroof *et al.* 1984). The tubes were incubated at 65°C for 60-90 min. After that, 4.5 ml of chloroform/octanol (24:1) was added to each tube and tubes were rocked to mix for 10 min, then centrifuged for 10 min at 3200 rpm. The supernatant was transferred to a new tube and 6 ml of isopropanol was added to each tube. After 60 min., the tubes were centrifuged for 10 min at 3200 rpm and the pellets obtained were dissolved in 400 µl of TE buffer (10 mM Tris-HCl, pH 8.0 + 1.0 mM EDTA). The DNA was stored at -20°C. Primer pairs were used to amplify segments of two *Cre* genes from the wheat genotypes (Majnik et al. 2003; Martin et al. 2004). Amplifications were performed in a thermal cycler (Thermolyne Amplitron) with 25 µl reaction volumes, containing 1 X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 unit of Taq polymerase supplemented with 0.01% gelatin, 0.2 mM of each dNTPs, 25 pmol of each primer and 50 ng of total genomic DNA. PCR products were separated by electrophoresis in a 1.5% agarose using Tris-acetate-EDTA (TAE) buffer and detected by ethidium bromide staining.

Statistical analysis

Combined data were subjected to analyses of variance (ANOVA) using SAS, 1985. Means were separated using Fisher's Protected $LSD_{0\cdot05}$ (SAS Institute, 1985). Correlation analysis among agronomical traits and number of cysts/root system was performed employing Pearson's test (Zar, 1984). Data from the SSR analysis were scored for cluster analysis on the basis of the presence or absence of the amplified products for each SSR primer. If a product was present in a cultivar, then it was designated as ''1''; and if absent, then itwas designated as ''0''. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to generate similarity coefficients based on the SIMQUAL module. The similarity coefficients were then used to construct a dendrogram by UPGMA (Unweighted Pair-Group Method with Arithmetical Averages) using NTSYS-PC software version 2.0 (Exeter Software, New York) (Rohlf, 2000).

Conclusion

There is a great deal of potential for this study to develop new resistant varieties to CCN which will have a great value to the agriculture sector in Saudi Arabia. Previous reports supporting the ability of *H. avenae* to reproduce on wheat cultivars worldwide are numerous (Meagher, 1977; Dhawan, 1988; Holdeman and Watson, 1977; Ibrahim, 1989). In Saudi Arabia, Al-Hazmi et al. (1994) reported the susceptibility of eight barley and wheat cultivars, including cv. Yecora Rojo to a local population of *H. avenae*. This makes the search for resistant or tolerant wheat cultivars of great necessity, and that was the main objective of this study. We could now use these resistant genotypes in our breeding programs to produce resistant wheat cultivars having also good agronomic traits.

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